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MANUAL OF METHODS IN AQUATIC ENVIRONMENT RESEARCH

Part 9 – Analyses of Metals and Organochlorines in Fish

with the cooperation of the
Swedish International Development Authority

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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Part 9. Analyses of metals and organochlorines in fish

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 1983

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PREPARATION OF THIS MANUAL

This Manual was prepared under the FAO/SIDA Government Cooperation Programme Project GCP/RAF/072/SWE, Seventh FAO/SIDA Workshop on Aquatic Pollution in relation to Protection of Living Resources.

DEFINITION OF MARINE POLLUTION

Pollution of the marine environment means: "The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".

IMO/FAO/Unesco/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP)

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PREFACE

Within the FAO/SIDA Cooperative Programme a series of workshops on aquatic pollution in relation to the protection of living resources was started in 1972. The seventh workshop was held in Manila, Philippines, hosted by the National Pollution Control Commission. It dealt with methods to analyse heavy metals and organochlorine compounds in molluscs, crustacea and fish, using atomic absorption spectrophotometry and gas-liquid chromatography. For the purpose of this workshop a manual was prepared covering the field sampling of aquatic organisms, the preparation of samples and the analysis of metals and organochlorines.

When drafting the manual, the existing one of Sampling and Analysis of Biological Material, prepared by M. Bernhard for use in the Mediterranean (Part 3 of this Manual) was duly taken into account. It was, however, felt that, for use at a training course for developing countries, the description of analytical methods should be more detailed, allowing the trainee to closely follow the instructions step by step. The choice of methods presented was adapted to the general conditions in laboratories in developing countries, methods that are as simple as possible and, at the same time, as accurate as necessary.

The manual was used successfully during the workshop in Manila and, based on the experience gained, some parts have subsequently been rewritten. The present version has been published to serve as a training tool for further workshops planned to be held in other regions under the FAO/SIDA Cooperative Programme. It is also hoped that this manual will serve as a laboratory guide in developing countries involved in monitoring of contaminants in their fishery resources.

Final editing of this manual was carried out by H. Naeve, of the Fishery Resources and Environment Division of FAO. The views expressed are those of the authors and do not necessarily represent the views of either FAO or SIDA.

Summary

This manual is describing analytical methods for measuring contamination of fish, crustacea and bivalves with heavy metals, organochlorines and methyl mercury. Chapter one describes field sampling, sample storage and preparation of subsamples of biological material for analysis. Chapter two describes the analysis of zinc, copper, lead and cadmium by flame atomic absorption spectrophotometry, and mercury and methyl mercury by flameless AAS. It includes descriptions of sample preparation, wet ashing with hydrogen peroxide/nitric acid, and the photometric measurement. Chapter three describes the analysis of organochlorines and methyl mercury by gas liquid chromatography. It covers the preparation of columns, extraction and fat isolation, clean up procedures by sulphuric acid and potassium hydroxide, the chromatographic determination and the calculation of results.

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1. FIELD SAMPLING AND PREPARATION OF SUBSAMPLES OF AQUATIC ORGANISMS FOR ANALYSES OF METALS AND ORGANOCHLORINES

compiled by

B. I. DYBERN

1.1 GENERAL REMARKS

1 Presumably all aquatic organisms are contaminated with one or more substances containing metal or *organochlorine* compounds which are directly or indirectly discharged into the natural waters by man. These substances, that may be toxic to man and to the ecosystem in very small concentrations (ppm or lower) may occur everywhere in nature and in the laboratory. Therefore, it is necessary to take such precautions that further contamination of the material during field sampling, preparation and analysis of aquatic organisms is avoided as much as possible.

2 For fish and shellfish the concentrations of the toxic substances are usually given per unit fresh weight and should preferably be expressed as mg/kg or - when the concentrations are very low - ng/kg. Dry weight expressions should be avoided since drying of biological material may cause escape of volatile fractions of the toxic substances. In the case of freeze-drying, the process itself may contaminate the material.

3 When monitoring contaminants in biological material, one should select the species according to the following criteria. They should be:

(1) easy to identify

(2) easily available in enough quantities

(3) as stationary as possible

(4) geographically widespread.

When it is possible to analyse several species these should represent different levels in the food chains, different systematic groups of organisms and types with high, as well as with low, fat content.

4 The number of specimens of each species in a sample should be enough for at least semi-statistical treatment of the results of the analyses, which means at least 10, preferably 15 or 20 individuals of the same size (age). The size should usually be the "normal", or commercial, adult size met in nature, unless the investigation aims at studying special development stages. If the monitoring continues through several years (to follow trends), one should always collect the same species and the same sizes, from the same sites and depths during the same time of the year.

5 Always the same methods and equipment should be used for field sampling, preparation of subsamples and analysis. Whenever possible, the same scientists and technicians should carry out the same steps of the investigations.

6 Fishes of 30-50 cm are easiest to handle and to take subsamples from. The generally more stationary mussels and oysters may give more precise information on the contamination of an area but the preparation of subsamples for analysis is more difficult. Shrimps, crabs and lobsters take an intermediary position in this respect.

7 All the specimens in a sample should preferably be analysed individually. This gives both the range and average of the concentration of the contaminants in question in a species. In many cases the analyses are very expensive. In such cases, the average concentration can be obtained by analysing a composite subsample which is taken from a homogenate of subsamples from several specimens (at least 10; up to 50 if easily available or very small). The range, however, is not obtained this way. This method can also be used when the animals investigated are too small to yield a subsample each. A subsample sent for analysis should weigh about 10 g (fresh weight).

8 If one is not absolutely sure about the species sampled, one or two specimens should be preserved in 70% alcohol or 10% formaline (equals 4% formaldehyde solution) for later systematic determination and for keeping as reference specimen.

1.2 FIELD SAMPLING AND PRESERVATION

9 Sampling gear should be suitable for collecting the desired species, with the lowest possible risk for contaminating and damaging the individual organisms. When possible, mussels and oysters should be carefully sampled by a diver to avoid that particles get inside the shells.

10 Selection of sampling sites should be done according to the known or expected presence of polluted and non-polluted areas. It is of advantage to make the sampling from research vessels at sea or at specialized field excursions to lakes and rivers. The sampling areas must be clearly identified and depth and general characteristics described (see 15). The same information must be recorded also when the specimens are purchased, e.g. on a fish market.

11 Field sampling should always be carried out by experienced people who know the importance of the precautions to be taken. Since the sampled animals must be frozen as soon as possible after catch, the sampling should not be carried out at a too long distance from freezing facilities. In tropical climates fish and crustacean shellfish, even if stored in ice, begin to decompose and to dry very soon after death. This causes loss of some of the compounds to be analysed. Purchase at fish markets should be made as soon as possible after the fish or crustaceans have been landed. Mussels and oysters can be stored a little longer provided that their shells are kept closed. They should be kept alive until freezing (see 16). In temperate and cold climates fish and crustaceans may be stored for several hours without loss of any detectable amount of the substances to be investigated.

12 The sampled animals may have to be transported from the sampling locality to a suitable, clean laboratory for further treatment. The transport should be carried out so that the animals are not damaged or contaminated. They should be kept as moist as possible but not submerged in water. The transport should be made in non coloured plastic buckets for animals destined to metal analysis and in *metal buckets* for those aimed at *organochlorine* analyses. Never store them in aquaria because the water is probably contaminated from plastic or metallic tubes or walls.

13 It is desirable to freeze all specimens as whole organisms. For instance, fish should not be degutted since this causes loss of fluid from different parts of the body. In the case of very big animals, however, such as tuna and swordfish, it is necessary to cut out pieces of appropriate parts of the body. In the protocol (see 15) it should always be noted whether the specimen was frozen whole or not.

14 Before freezing, the sampled specimens should be cleaned, weighed (to nearest 1 g for bigger ones and 0.1 for smaller ones), and measured (to nearest 1 cm for bigger ones and 0.1 cm for smaller ones). The whole treatment of the specimens from catch to freezing should be done with care and in an environment as clean as possible, away from contamination sources such as smoke, oil, bottom paints on ships, etc. All animals with damaged skin or broken shell should be discarded since they may have been further contaminated during handling.

15 Before freezing, the specimens must be labelled and packaged. The labels should carry necessary information for subsequent identification, such as date, locality, depth and code number. The information listed below should be recorded in a notebook destined to this purpose (do not use loose sheets of paper which may get lost) (see also 24):

(a) Number or designation of sampling or expedition

(b) Code number of specimen for individual analysis or sample for composite analysis

(c) Date of collection

(d) Position of sampling locality, preferably as geographical coordinates, and depth

(e) Special characteristics of the sampling locality, including pollution

(f) Sampling method used

(g) Total length of specimen for individuals. Length range and average for sample for composite analysis. Number of specimens, in the latter case

(h) Weight of specimen for individual analysis. Weight range and average for sample for composite analysis. Number of specimens

(i) Storage method before preparation. Time before freezing

(j) Type of analysis

(k) Name of person reponsible for field sampling and packaging

(l) Special notes, e.g. size of catch, deviations from normal sampling procedure.

16 Packaging procedures: The packaging can be done in slightly different ways, depending on the size of the animals and on the kind of analysis they will be prepared for. It is best done in connection with weighing and measuring of the specimens.

Samples destined for analysis of metals shall not get in contact with any metallic instruments; samples for analyses of *organochlorines and methyl mercury* shall not get in contact with plastic material. The descriptions are made for samples for metal analyses. Instruments and materials to be used for samples for *organochlorine and methyl mercury* analyses are indicated in *italics*.

Bigger fish and crustaceans

1. Discard damaged specimens. Wash each individual in clean, natural sea or fresh water (depending on whether they are marine or freshwater species). Avoid tap water if possible, since it may contain metals.

2. Weigh and measure the animals and record the values (see 14 and 15).

3. Put each individual in a plastic bag/*aluminium foil* and roll the plastic bag to squeeze out the air. Be careful that the finrays or the spiny parts of the crustaceans do not make holes in the bag/*foil*.

4. Put the plastic bag/*foil* with the animal into another plastic bag and put a label in the outer bag. Roll to squeeze out the air and put a rubber band around. The label will be kept dry in this way.

5. Put all the plastic bags containing specimens from the same sample into a big plastic bag or a box, with a label inside. Transport to a freezer.

Smaller fish and crustaceans for composite analyses

These specimens are too small for individual analyses (give less than 10 g muscle tissue from required part of the body) and will be used for homogenized subsamples (see 7).

1. Discard damaged specimens. Wash each individual in clean, natural sea or fresh water (depending on species). If possible avoid tap water.

2. Weigh and measure the animals and record the average values and ranges (see 14 and 15).

3. Put the measured number of animals into a plastic bag/*aluminium foil* and roll the plastic bag to squeeze out the air. Check that the sharp parts of the specimens do not make holes in the bag/*foil*. In case of very spiny animals, one might consider wrapping each animal in a bag/*foil* before putting them in the composite sample bag/*foil*.

4. Put the plastic bag/*foil* into another plastic bag and put the label into the latter before rolling it to squeeze out the air. Seal with a rubber band and transport to freezer.

Molluscs, bigger and smaller

Are treated in the same way, independently of whether they will be used to prepare individual or composite subsamples.

1. Discard damaged specimens and clean the rest from sessile flora and fauna. Cleaning should be done in running water with the shells well closed.

2. Put the mussels or oysters in clean, natural, standing sea or fresh water (depending on the species). The temperature should be about the same as at the sampling site. Keep them in water for 12-24 hours to make them rinse

their gills from particles and their intestines from faecal matter. The container holding the water should be of non-coloured plastic material/ *metal or glass*.

3. Place a small plastic/*wooden* stick between the valves of the molluscs to prevent them from closing. Take them out of the water and allow the internal water to flow out. If necessary, use plastic/*steel* knives to open the shells.

4. Weigh and measure the animals and record the values for each specimen when aiming at individual analyses, and average values and ranges when aiming at analyses of composite samples (see 14 and 15).

5. Put the animals in individual plastic bags/*aluminium foil* if aimed at individual analyses. Put the plastic bags/*foils* into other plastic bags with a label in the outer ones. Squeeze out the air and seal with a rubber band. Put all the bags into a big bag or box. Label and transport to the freezer. In case of animals aimed at composite analyses, put all individuals belonging to the same sample in a plastic bag/*aluminium foil*. Squeeze out the air, put the plastic bag into another one and the label into the latter. Seal with a rubber string and transport to freezer.

17 After packaging, the material should be transported to the freezer as quickly as possible, preferably within a few minutes. Freezing and subsequent storage should be done at a temperature of -18°C (0°F). If too much material is put into the freezer at the same time the temperature may rise above this value. That should be avoided since the freezing process will be slowed down, thus permitting bigger ice crystals to be formed, which might cause damage to tissues. It may, therefore, be of advantage to do the packaging near the freezer and to put the packed specimens successively into it. The freezer should be put on maximum power shortly before, during and shortly after the freezing process.

18 After use, all laboratory and other equipment should be carefully cleaned, preferably with detergents, and rinsed with running tap water.

1.3 PREPARATION OF SUBSAMPLES FOR ANALYSIS

19 Provided the temperature is low enough, the frozen fish and shellfish may be stored in the freezer for half a year or more. But since they occupy space and new material may successively come in, it is of advantage to prepare subsamples as soon as possible. The subsamples may have to wait for a long time before they are analysed, but since they are small, they do not take much space.

20 When preparing the subsamples one should be very careful not to contaminate them, bearing in mind that the quantities of contaminants are very small (ppm or smaller); they will be registered during the analyses, and it is easy to change these figures considerably by making mistakes. The preparations should thus be carried out in a clean and dust-free laboratory, and all equipment and instruments should be carefully cleaned immediately before and after use (see 23).

21 For the final analyses with atomic absorption spectrophotometer and *gas chromatograph* the analyst needs subsamples of about 10 g from each individual fish or shellfish, or from a homogenate from several specimens (see 4 and 7). It is generally up to the biologist in charge of the field sampling to prepare these subsamples.

22 Preparation procedures

Samples destined for analyses of metals shall not get in contact with any metallic instruments; samples for analyses of *organochlorines and methyl mercury* shall not get in contact with plastic material. The descriptions are made for samples for metal analyses. Instruments and material to be used for samples for *organochlorine and methyl mercury* analyses are indicated in *italics*. Instruments are changed according to the following table:

Equipment	For analyses of: Metals	For analyses of: *Organochlorines and methyl mercury*
Knives	Plastic or glass	*Steel*
Tweezers	Plastic	*Steel*
Scissors	-	*Steel*
Plates	Glass or plastic	*Glass or steel*
Tubes and jars	Plastic	*Glass*
Lids	Plastic	*Plastic lined with aluminium foil*

Bigger fish specimens

1. Take the fish from the freezer and keep it at room temperature until it is partly thawed. Unwrap it.

2. Put the fish on a clean glass or plastic plate/*glass or steel plate* with the left/*right* side upward, nose to the left/*right* (Fig. 1). This is a standard procedure for handling the fish always in the same way.

3. Remove the skin with a plastic or glass knife/*steel knife* and plastic/*steel* tweezers from the lower part of the dorso-lateral muscle, in the middle of the fish, caudal fin not counted (Fig. 1).

4. Cut a subsample of the muscle of about 10 g and put it in a plastic/*glass* tube or small plastic/*glass* jar on a tared balance. The cutting is easier when the fish is half-thawed.

5. Weigh the piece of muscle tissue with an accuracy of 0.1 g and record the weight in the notebook.

6. Put the lid onto the tube or jar, label it or engrave the code number of the fish. Note that the label must be on the outside and firmly attached. *For organochlorine analyses, place a piece of aluminium foil between the glass tube or jar and the lid.*

7. Put the tube or jar together with the tubes or jars belonging to the same sample in a box, label it and keep it in the freezer.

8. Clean the equipment used.

Note: If the fish is small but still of a size of more than about 15 cm total length, it is still possible to prepare an individual subsample by using more or less the whole fillet on left/*right* side.

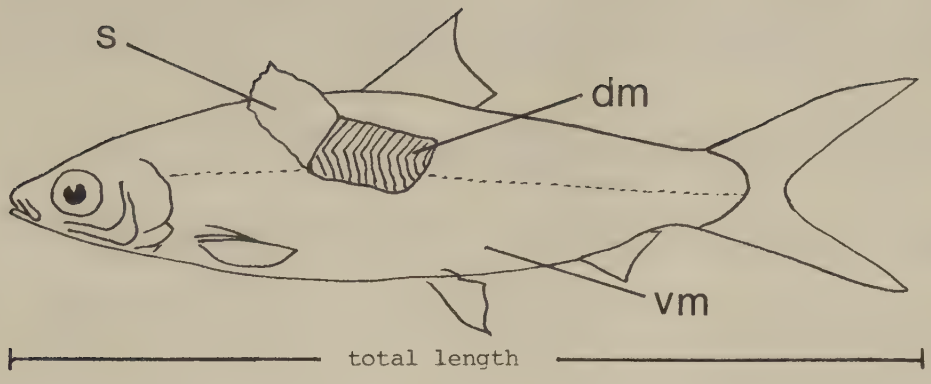


Figure 1. Dissection of a fish
s - skin being removed; dm - dorsolateral muscle; vm - ventrolateral muscle

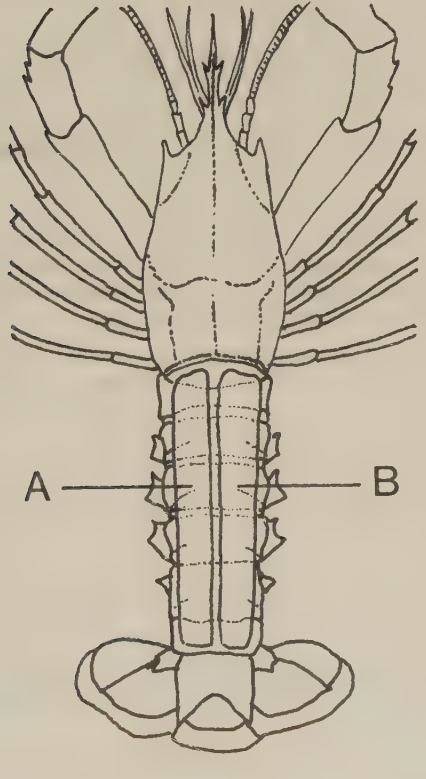


Figure 2. Dissection of crustacea
A - Muscular tissue on left side is taken for analyses of metals
B - *Muscular tissue on right side is taken for analyses of organochlorines and methyl mercury*

Smaller fish specimens (less than about 15 cm total length)

The muscle tissue is not enough for preparing individual 10-g subsamples. Pieces of the lateral muscles (or the whole fillets) of several specimens are instead put together and homogenized. The subsample is taken from the homogenate.

1. Take the fishes from the freezer and keep them at room temperature until they have partly thawed. Unwrap them.

2. Put the fishes on a clean glass or plastic plate/*glass or steel plate* with the left/*right* side upward and nose to the left/*right* (Fig. 1).

3. Remove the skin on the left/*right* side and cut out equal pieces of the lateral muscles from all the fishes, preferably in the middle part of the body.

4. Put the muscle pieces on small, clean plastic/*aluminium* foil and weigh them. Try to obtain pieces of approximately the same weight.

5. Cut the pieces into smaller pieces with a plastic or glass knife/*steel knife*, mix the smaller pieces until there is a homogeneous mass. For homogenization one can also make use of a glass, quartz or teflon mortar. Grinding must then be carried out very carefully so that particles from the mortar do not contaminate the sample. *For organochlorine analyses, motor-driven homogenizers may be used, if they have no plastic parts and no grease leaks from them onto the sample.*

6. Take a subsample of about 10 g from the homogenate and put it in a plastic/*glass* tube or small plastic/*glass* jar on a tared balance.

7. Weigh the subsample with an accuracy of 0.1 and record the weight in the notebook.

8. Put the lid onto the plastic/*glass* tube or jar; label it or engrave with the code number of the fish sample. The label must be on the outside and firmly attached. *For organochlorine analyses, place a piece of aluminium foil between the glass tube or jar and the lid.*

9. Put the tube or jar into the freezer.

10. Clean the equipment used (see 23).

Bigger crustaceans (of such size that half the abdominal muscle provides a subsample of at least 10 g. Fig. 2)

1. Take the crustacean out of the freezer and let it thaw almost entirely. Unwrap it.

2. Put the crustacean on a clean glass or plastic plate/*glass or steel plate*, ventral side down.

3. Remove the abdominal plates from the animal (do not use metal scissors in case of metal analyses!).

4. Cut out a piece of about 10 g from the abdominal muscle on the left/*right* side of the abdomen. Leave a thin wall of muscular tissue in the middle of the abdomen (see Fig. 2) to protect the right/*left* side from being contaminated by the instruments used. (Remove the intestine if adhering to the subsample). Put the subsample into a plastic/*glass* tube or jar on a tared balance.

5. Weigh the piece of muscle with an accuracy of 0.1 g and record the weight in the notebook.

6. Put the lid onto the plastic tube or jar, label it or engrave the code number of the animal. The label must be on the outside and firmly attached. *For organochlorine analyses, place a piece of aluminium foil between the glass tube or jar and the lid.*

7. Put the tube or jar together with the other subsamples from the same sample in a box, label it and keep it in the freezer.

8. Clean the equipment used (see 23).

Smaller crustaceans

Muscle pieces from the abdomen of all the specimens belonging to the same sample are put together and homogenized. Note: the muscle tissue of the whole abdomen must usually be used.

1. Take the crustaceans out of the freezer and let them thaw almost entirely. Unwrap them.

2. Put the crustaceans on a clean glass or plastic plate/*glass or steel plate*.

3. Remove the abdominal plates from the animals (do not use metal scissors for metal analyses.

4. Cut equal pieces of abdominal muscle tissue from all the animals. Remove the intestines as much as possible.

5. Put the muscle pieces on small, clean plastic/*aluminium* foil and weigh them. Try to obtain pieces of approximately the same weight.

6. Cut the pieces into smaller pieces with a plastic or glass/*steel* knife, mix the smaller pieces until there is a homogeneous mass. For homogenization one can also make use of a glass, quartz or teflon mortar. Grinding must then be carried out very carefully so that particles from the mortar do not contaminate the sample. *For organochlorine analyses, motor-driven homogenizers may be used, if they have no plastic parts and no grease leaks from them onto the sample.*

7. Take a subsample of about 10 g from the homogenate and put it in a plastic/*glass* tube or jar on a tared balance.

8. Weigh the subsample with an accuracy of 0.1 g and record the weight in the notebook.

9. Put the lid onto the plastic/*glass* jar or tube, label it or engrave with the code number of the sample. The label must be on the outside and firmly attached. *For organochlorine analyses, place a piece of aluminium foil between the glass tube or jar and the lid.*

10. Put the tube or jar into the freezer.

11. Clean the equipment used (see 23).

Bigger molluscs (soft parts of the whole individuals must weigh 10 g or more)

It is generally impossible to take subsamples from e.g. muscular tissue. Instead one has to homogenize the soft parts of the whole animal and take the 10-g subsample from the homogenate.

1. Take the specimen out of the freezer and let it thaw completely. Unwrap it.

2. Open the shell carefully without breaking it by means of a plastic/*steel* knife. Put the animal on a clean linen with the opening downward and let the excess water drop out for about 10 minutes.

3. Weigh the animal and note the weight in the notebook.

4. Open the shell as wide as possible. Cut out carefully the soft part with a plastic/*steel* knife and put it on a piece of plastic/*aluminium* foil or a watch glass. Take away byssus or fastening threads, if any.

5. Under a stereo microscope remove with plastic/*steel* tweezers particles, pieces of shell, etc., from the soft parts, if any.

6. Weigh the soft part of the animal and record the weight in the notebook.

7. Cut the pieces into smaller pieces with a plastic or glass knife/*steel knife*, mix the smaller pieces until there is a homogeneous mass. For homogenization one can also make use of a glass, quartz or teflon mortar. Grinding must then be carried out very carefully so that particles from the mortar do not contaminate the sample. *For organochlorine analyses, motor-driven homogenizers may be used, if they have no plastic parts and no grease leaks from them onto the sample.*

8. Take a subsample of about 10 g from the homogenate and put into a plastic/*glass* tube or jar on a tared balance.

9. Weigh the subsample and record the weight in the notebook.

10. Put the lid onto the tube or jar, label or engrave the code number of the animal. The label must be on the outside and firmly attached. *For organochlorine analyses place a piece of aluminium foil between the glass jar or tube and the lid.*

11. Put the tube or jar together with the other subsamples from the same sample in a box. Label the box and keep it in the freezer.

12. Clean all equipment used (see 23).

Smaller molluscs

The soft parts from a number of individuals (10-15) have to be used to form a homogenate from which a subsample to be analysed is taken.

1. Take the specimens out of the freezer and let them thaw completely. Unwrap them.

2. Open the shells carefully without breaking them by means of a plastic/*steel* knife. Put the animal on a clean linen with the opening downward and let the excess water drop out for about 10 minutes.

3. Weigh the animals and record the mean weight in the notebook.

4. Open the shells as wide as possible. Cut out carefully the soft parts with a plastic/*steel* knife and put them on a piece of plastic/*aluminium* foil or a watch glass. Take away byssus or fastening threads if any.

5. Under a stereo microscope, remove with plastic/*steel* tweezers particles, pieces of shell, etc., if any, from the soft parts.

6. Weigh the soft parts of the animals. Make sure that all soft parts used for the composite sample have approximately the same weight.

7. Cut the animals into smaller pieces with a plastic or glass knife/*steel knife*, mix the pieces until there is a homogeneous mass. For homogenization one can also make use of a glass, quartz or teflon mortar. Grinding must be carried out very carefully, so that particles from the mortar do not contaminate the sample. *For organochlorine analyses, motor-driven homogenizers may be used, if they have no plastic parts and no grease leaks from them onto the sample.*

8. Take a subsample of about 10 g from the homogenate and put it into a plastic/*glass* tube or jar on a tared balance.

9. Weigh the subsample and record the weight in the notebook.

10. Put the lid onto the tube or jar, label or engrave the code number of the sample. The label must be on the outside and firmly attached. *For organochlorine analyses, place a piece of aluminium foil between the glass tube or jar and the lid.*

11. Put the tube or jar into the freezer.

12. Clean all equipment used (see 23).

23 Cleaning of the equipment: Clean equipment should be used for each subsample of individual animals in order not to trasfer contamination from one subsample to another. It is not so necessary to clean all equipment between the many smaller individuals contributing to the same homogenized composite subsample, but it must be done between different composite subsamples. Be sure that there are always reserve instruments available if something happens to the ones used.

Cleaning procedures for equipment for metal analyses

Plastic and glass knives, plastic tweezers, plastic or glass plates, plastic sticks (for molluscs)

1. Wash in tap water with detergent.

2. Wash in running tap water.

3. Store smaller items for some minutes in concentrated HNO_3:H_2O=1:6. Wash glass plates, etc.

4. Wash with quartz-distilled water (ordinary distilled water may contain metals from the distillation apparatus).

5. Dry on clean linen in dust-free air.

Plastic tubes or jars and plastic lids

1. Always use new ones.

2. Before use, wash with quartz-distilled water.

3. Dry on clean linen in dust-free air.

Cleaning procedures for equipment used for analyses of organochlorine substances and methyl mercury

Steel knives, tweezers and scissors, metal or glass plates

1. *Wash in tap water with detergents.*
2. *Rinse in running tap water.*
3. *Wash with concentrated* HNO_3:H_2O=1:6.
4. *Wash with distilled water.*
5. *Rinse with acetone, preferably redistilled, or ethanol.*
6. *Dry on clean linen in dust-free air.*

Glass tubes or jars

1. *Store for about 60 minutes in hot tap water containing a strong detergent.* *Note: do not use plastic containers.*
2. *Wash in tap water.*
3. *Wash with distilled water*
4. *Dry on clean linen in dust-free air.*

24 Recording

Information in addition to that listed before in a special notebook (see 15) and obtained during the preparation of the subsamples, and also that received after the analyses, can be recorded, preferably in the same notebook.

(m) Date of preparation of subsample.

(n) Number of individuals in homogenized subsample.

(o) Weight of subsample.

(p) Sex, sexual development stage (if possible).

(q) Age (if possible).

(r) Date of despatch of subsamples for analyses.

(s) Name of laboratory and scientist in charge of analyses.

(t) Date of analyses.

(u) Fat content of subsample.

(v) Date of receipt of results of analyses.

(w) Concentrations of substances analysed in muscle tissue.

(x) Concentrations of substances analysed in extracted fat.

(y) Special notes, e.g. on preparation of other parts of the animal, storage of excess material.

25 It may be necessary to have the analyses carried out in another laboratory than the one where the preparation of subsamples has taken place. Transport of the subsamples must be effected in such a way that they are maintained frozen all the time, and, upon arrival at the laboratory, they should immediately be put into the freezer until the analyses can be done. Since a laboratory with access to sophisticated equipment such as atomic absorption spectrophotometer and gas chromatograph can also, presumably, afford good freezing facilities, it is often good practice to send the subsamples to the analytical laboratory as soon as possible after preparation for storage there.

26 It is recommended to prepare duplicate subsamples whenever possible. One of the duplicates may serve as a reserve if something happens to the other one. It may also be saved for analyses with future improved techniques or used for intercalibration exercises between laboratories. Duplicate subsamples must be prepared from the same part of a fish or shellfish or from a well-homogenized composite sample.

27 As mentioned in 22, subsamples from fish and crustaceans for analyses of metals are taken from the left side of the animals, while subsamples for analyses of *organochlorines* are taken from the *right* side. This means that one specimen can be used for preparing both kinds of subsamples, and that direct comparison of the concentrations in the same animal can be done. When using the same animal in this way one should start by preparing the left-side subsample first and try not to damage the other side. From the *right* side one can take extra samples if one intends to investigate also *methyl mercury* by means of gas chromatography. It is not allowed to take subsamples for both analyses of metals and of *organochlorines* from a homogenate, since, due to the preparation and homogenization processes, it is contaminated by either metals or by *organochlorines*. However, extra subsamples for analyses of *methyl mercury* may be taken from the same homogenate as the subsample for analyses of *organochlorines*.

28 The liver is the detoxification organ of the body and it tends to accumulate toxic substances. Results of analyses of the liver of fish can sometimes give a clearer picture of the contamination than results of analyses of muscular tissue. Liver subsamples can be prepared in accordance with what has been said about muscular tissue; however, they should, if possible, be from the inner part of the liver.

29 Fat also accumulates more contaminants, for example *organochlorines*, than most other tissues. When muscle tissue is analysed for the latter, the fat of the muscle is automatically extracted and analysed.

30 It is possible to use fresh fish and shellfish for preparation of subsamples without prior freezing. It is, however, strongly recommended to freeze the animals first if facilities are available. To cut out pieces of fresh fish tissues with a plastic or glass knife can be very difficult. Heavy losses of body fluid can alter the composition of the tissues to be prepared and, consequently, the results of the analyses.

2. ANALYTICAL METHODS FOR MEASURING METALS BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

compiled by

J. DALZIEL and C. BAKER

2.1 OUTLINE

31 Typical ranges for the concentrations of most of the important trace elements in marine organisms are shown in Table I, expressed as nanograms (10^{-9}) per gram fresh weight of sample (ng/g FW). These concentrations are high enough for the determination of many of the elements (Zn, Cu, Cr, Mn, Pb, Cd) by simple flame atomic absorption spectrophotometry (AAS). This is generally the method of choice at the present time. It involves aspiration of a solution of the sample into the air-acetylene flame, after destruction of the bulk of the organic matter by 'wet ashing' with a mixture of nitric acid and hydrogen peroxide. No other chemical treatment is required. The detection limit (DL) for each element by flame AAS is also shown in the table, in nanograms per millilitre (ng/ml). The comparison gives guidance on the dilution that can be tolerated in the wet ashing step, prior to the AAS determination.

Table I

Typical concentration ranges of trace elements in aquatic organisms and their detection limits in flame atomic absorption spectrophotometry

Element	Range in organisms (ng/g FW)	Detection limits by AAS (ng/ml)
Zn	3 000 - 100 000	1 - 3
Cu	200 - 50 000	1 - 8
Cr	200 - 10 000	3 - 8
Mn	100 - 20 000	2 - 8
Pb	50 - 5 000	10 - 30
Cd	10 - 1 500	2
Se	500 - 2 000	
As	200 - 10 000	
Hg	200 - 3 000	

32 The elements Zn, Cu, Pb, Cd will be determined in samples of fish, shrimp and oyster, along with blanks and standard material, using the procedures described below.

33 Modifications of the basic AAS method are needed for the determination of Hg, As and Se. Mercury is of particular importance because it can occur in the environment in both inorganic and organic forms. The latter is extremely toxic. Total mercury is determined by a 'cold vapour' technique. After wet

ashing, the total mercury (as Hg^{2+}) is reduced to the metallic state when it can be volatilized by aeration and measured by flameless AAS. The determination of organic mercury alone can be carried out on a separate sample using either gas chromatography or flameless AAS again. The first step for both procedures requires the selective complexation of the organic mercury by cysteine followed by its separation from the inorganic mercury by partition into an organic solvent.

34 Total and inorganic mercury will be determined in the samples with this 'cold vapour' technique using the procedures described below.

35 There are two important advantages in keeping the procedures for the determination of trace elements in aquatic organisms as simple as possible. These are:

(i) By reducing the need to handle the samples and keeping the use of chemical reagents to a minimum, the risk of contamination is kept low. Great care is still needed, however, and contaminated glassware and impure reagents must be avoided. A strict regime of laboratory cleanliness is most important, and it is necessary to perform 'blank' determinations and analyse standard materials in order to check accuracy and precision (if possible, on the basis of inter-laboratory tests).

(ii) These methods require the minimum apparatus and operator time per sample, so that they can be applied to large batches of samples in routine analysis for broad surveys of the pollution levels in aquatic organisms. Such tests must be carried out on the same organisms taken over wide areas before valid comparisons of aquatic pollution can be made.

36 The procedures that form this part of the manual are designed to help the establishment of regional programmes for monitoring aquatic pollution from trace elements.

2.2 HEALTH AND SAFETY PRECAUTIONS

37 Hydrogen peroxide is a potentially hazardous material. It may cause fires if spilled onto easily-combustible objects and contact with the skin should be avoided. If spills occur, they should be diluted with copious amounts of water and mopped-up carefully (do not use paper tissues). Do not add hydrogen peroxide directly to dried biological material. For the analysis of dried materials treat first with nitric acid alone, until reaction has ceased, and only then should hydrogen peroxide/nitric acid be used. Safety glasses should be worn at all times when handling hydrogen peroxide and when near fume hoods used by others for this purpose.

38 Acetylene/air mixtures are a potential explosion hazard unless careful attention is paid to the instrument manufacturers' instructions. The extraction hood over the flame must be used as the combustion products are toxic and corrosive. When using acetylene/air flames remember, when switching on, turn on air first, when switching off, turn off acetylene first. The acetylene should be turned off in the event of a power cut even if there is an automatic cut-off of the instrument.

2.3 PROCEDURES EMPLOYED

39 For Zn, Cu, Pb, Cd, the following steps are to be carried out:

- Preparation of standards of the trace elements
- Construction of calibration plots, after learning the use of the flame AAS
- Wet ashing the biological material and determination of the trace elements
- Analysis of standard materials and assessment of errors.

40 For total and organic Hg, the steps to be carried out are:

- Preparation of standards of total and organic mercury
- Construction of calibration plots, after learning the use of the 'cold vapour' instrument
- Solvent extraction of organic mercury and conversion to the ionic form
- Analysis of samples and standards and assessment of errors.

2.4 PREPARATION OF STANDARDS OF Zn, Cu, Pb, Cd AND Hg

41 Preparation of standard solutions (1 000 µg/ml)

Dissolve 100 mg of each of these elements separately in a minimum of distilled water, in 100-ml volumetric flasks, and dilute to the mark with 5% v/v nitric acid. When making up standard solutions it is best, both for purity and accuracy, to use purified metals. If metal salts are used, as here, these must be of analytical grade with known impurity limits. It is best to use a fresh bottle, and avoid salts that are hydrates, especially ones which effloresce or deliquesce. For weighing the substances, an analytical balance must be used.

42 Cross-contamination is the great danger in any trace element analysis. It occurs if the samples are allowed to come in contact in any way even with small amounts of much higher concentrations of the element which is to be determined. Good laboratory practice is essential to ensure that this cannot happen.

43 Preparation of working solutions

1. Dilute the standard solutions with 5% nitric acid to a concentration about ten times that which is to be determined in samples, using a pipette and clean volumetric flask.
2. Rinse a clean burette with this solution and fill to the mark.
3. Dispense measured aliquots of solution (say 2, 4, 6, 8 and 10) into small volumetric flasks (25 ml) in order to cover the working range for AAS.
4. Make up to the mark with 5% nitric acid as before and do not store these very dilute solutions for more than a day or so.
5. Record the concentrations of these in ng/ml.

44 Organic mercury standards (100 µg Hg/ml)

Dissolve 151 mg methyl mercuric dicyandiamide or an equivalent of another compound in a litre of 1% cysteine hydrochloride.

2.5 CONSTRUCTION OF CALIBRATION PLOTS, INCLUDING NOTES ON APPARATUS FOR AAS

45 Switch on the atomic absorption spectrophotometer, adjust to correct sensitivity and use this instrument according to the manufacturers' instructions. Record the absorbance of each working solution in turn, using a wash of distilled water to return the flame to a steady zero between readings. Use the results to construct the calibration plot of absorbance (relative units) against concentrations of working solutions (ng/ml) for each element (Fig. 3).

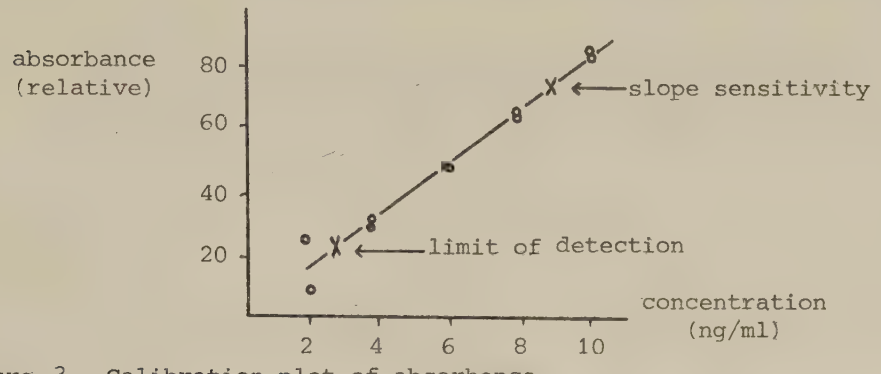


Figure 3. Calibration plot of absorbence

There is a linear relationship between absorbance

$$\log \frac{1}{\text{transmittance}}$$

and concentration because atomic absorption obeys Beer's law. The terms 'limit of detection' (LD) and 'slope sensitivity' (absorbance units/ng/ml) are illustrated on the diagram.

46 The solutions of the biological materials are aspirated into the flame under identical conditions and the concentration in ng/ml in the solutions are then read directly from the calibration plot. These concentrations must be multiplied by a dilution factor in order to determine the total amount of the element in the original samples.

Example: If 25 ml of solution (v) containing 3 g FW of sample (w) reads out with the concentration (c) of 5 ng/ml in the AAS, the amount of the element (x) in the sample is

$$x = \frac{c \cdot v}{w} = \frac{5 \cdot 25}{3} \approx 42 \text{ ng/g FW}$$

47 The wavelengths of the most sensitive lines from the hollow cathode lamps (HCL) are:

Element	Zn	Cu	Pb	Cd	Hg
Analysis line (nm)	213.9	324.7	217.0 (283.2)	228.8	253.7

48 Errors due to non-atomic absorption of the HCL line source will occur due to light scattering, especially with samples such as those containing salts from grams of biological materials (Na, K, Ca, Mg, Cl, PO_4). It is essential that a background correction should be applied to all readings from unknowns of this kind. This can be done either by chemical or instrumental corrections, thus,

(a) The working standards can be made-up to contain equivalent amounts of Na, K, Mg, Ca, Cl, PO_4 so that the light scattering will be the same in samples and standards, and an ordinary single beam AAS can be used.

(b) The reading of the absorption of the HCL line by both atomic and non-atomic processes in the sample flame can be compared with the absorption of light from a deuterium lamp (continuous source) at the same wavelength by the non-atomic process alone. This can be done automatically on the same sample using a modern double-beam AAS which subtracts one signal from the other and when available is the method of choice.

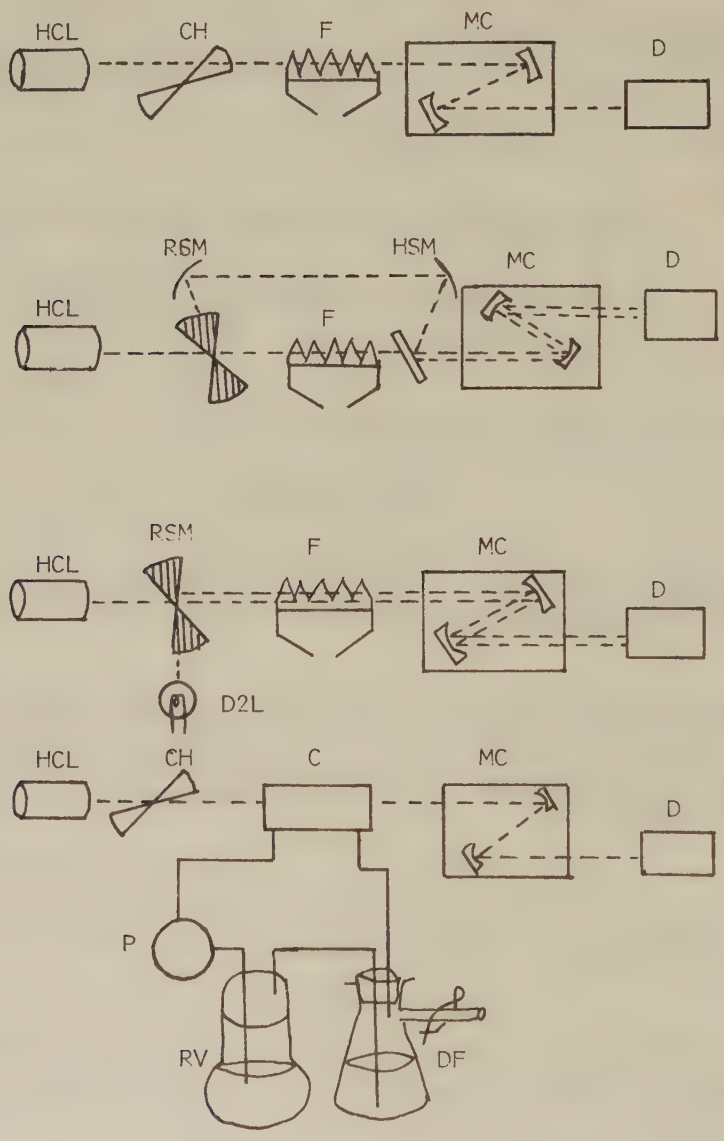


Figure 4. Diagrams of systems used in the AAS
C - cell; CH - chopper; D - detector; D2L - deuterium lamp; DF - drying flask; F - flame; HCL - hollow cathode lamp; HSM - half silvered mirror; MC - monochromator; P - pump; RSM - rotating sector mirror; RV - reaction vessel

49 The diagrams (Fig. 4) show a variety of single- and double-beam systems used in AAS. There is also the apparatus for the flameless AAS of mercury; some times the absorption cell will fit into a single-beam instrument.

50 The sample containing Hg^{2+} is placed in the reaction vessel (R) and there reduced with stanneous salts to elemental mercury. The solution is aerated and the moist mercury vapour passed through a drying flask (F) to remove water and on into the absorption cell for flameless AAS. From there it is returned via the pump and round this closed system until a constant reading is obtained on the recorder.

51 There are also open systems and some which involve preconcentration on gold gauze, where the response is measured as a peak and the limit of detection can be improved.

2.6 WET-ASHING OF BIOLOGICAL MATERIAL FOR DETERMINATION OF TRACE ELEMENTS AND RECOVERY OF ORGANIC MERCURY FOR AAS

52 1. Remove the sub-samples from the deep-freezer and allow time for them to thaw before opening the plastic tubes.

2. Open each tube and transfer the whole sample of about 10 g that was weighed previously, into a clean 100-ml beaker.

3. Add 10 ml of freshly prepared 1 : 1 v/v hydrogen peroxide/nitric acid, prepared in a small measuring cylinder as required, and cover the beaker with a watchglass.

4. Set the beaker aside, for about an hour, until the first reaction subsides.

5. Place the beaker on the hot-plate and carefully allow the temperature to rise to 160°C.

6. Continue to boil gently for about two hours and reduce the volume to between 2-5 ml. Any reaction should have ceased but do not allow the sample to go dry or it must be discarded.

7. Allow the solution to cool, transfer to a volumetric flask (25 ml) and dilute to the mark with distilled water (i.e. the test solution will be about 4-10% v/v nitric acid and not more).

53 Blanks and known amounts of the working standard solutions should be taken through this wet-ashing procedure to test for methodic and operative errors (see next section).

54 A great advantage here is that this simple wet-ashing procedure is sufficient to solubilize all of the trace elements and destroy most of the organic matter, which then do not interfere with the determination. Earlier methods required the complete destruction of the organic matter and it was necessary to use perchloric acid in the final stages. This can be extremely dangerous and has been the cause of serious explosions even when used correctly.

55 The total mercury will be determined after wet-ashing the organic matter with hydrogen peroxide and nitric acid as described above. For organic mercury follow this procedure:

1. Take an aliquot of the aqueous cysteine hydrochloride solution which has been extracted from the samples used to determine methyl mercury by GLC (see 92).

2. Dilute to about 10 ml with water.

3. Add 1.0 ml sulphuric acid and 2 ml of 10% v/v ammonium persulphate.

4. Heat slowly to boiling and continue for a minute or two in order to remove most of the excess persulphate.

5. Cool, transfer to a volumetric flask (say 25 ml) and dilute to the mark.

6. Proceed with the determination of mercury by flameless AAS in exactly the same way as for the total mercury.

2.7 ANALYSIS OF STANDARD MATERIALS AND THE ASSESSMENT OF ERRORS

56 In general, no analytical result is acceptable unless it can be accompanied by a 'confidence level' showing the range that would most probably include the result and the 'true value'. Knowledge of the confidence level of results expected from these procedures can be obtained in the following ways:

(i) An experimental study can be made of the errors that arise at each step in the procedure. These may arise from operative, methodic and instrumental causes and may be either random or systematic in their effects, i.e. the former giving the 'spread' and the latter the 'bias' of the results obtained from the true value. An example here is that the wet ashing and evaporation on the hot plate is most likely to cause operative and methodic errors. These should be tested because the results so far are biased because the AAS calibrations have not been taken through the full wet ashing procedure. It is recommended to take duplicate standards and blanks through the whole procedure and compare these results with duplicates read directly on the AAS, and to note the error as spread and bias expected, compared with the calibration curve.

(ii) The procedures can be tested using standard materials for which others have reported 'known values' or for which analyses have been made by other 'standard methods'.

57 When a large number of results of the same kind are available, they are best reported with statistical limits. The data must refer to the same 'consignment', as for example when the same species has been analysed from a specified area on a regular basis, as would be the situation in regional programmes for monitoring the aquatic pollution from trace elements. The first step toward this would be to determine the 'variance' (V) of a series of replicate results, x_1, x_2, x_3, ...x_i, ...x_n. This is $V = s^2$ where the 'standard deviation' (s) of these results is

$$s = \sqrt{\frac{1}{n-1} \Sigma (x_i - \bar{x})^2}$$

With $\bar{x}$ as the 'mean' of these results

$$\bar{x} = \frac{1}{n} \Sigma x_i$$

and to compare the values of $\bar{x}$ and s, it is useful to report the 'coefficient of variation' (CV), thus

$$CV = 100 \frac{s}{\bar{x}}$$

which is sometimes called 'relative standard deviation' of the results. For the procedures described here, we shall hope to be better than ±10%.

3. ANALYTICAL METHODS FOR MEASURING ORGANOCHLORINES AND METHYL MERCURY BY GAS CHROMATOGRAPHY

compiled by

S. JENSEN, L. REUTERGÅRDH and B. JANSSON

3.1 THE GAS CHROMATOGRAPHIC COLUMN

58 A chromatographic system consists of a mobile and a stationary phase. The separation of two compounds can be obtained if their partition between the two phases is different. In gas chromatography (GC), the mobile phase is a gas and the stationary phase can be a solid (GSC), but in the analyses of organic compounds it is normally a liquid (GLC).

59 Liquid stationary phases can be applied on the wall in a narrow tube (capillary column) or on an inert support material which is then filled into a wider tube (packed column). The capillary columns give better resolutions than the packed ones, but are difficult to prepare and to handle. Most analyses of chlorinated pesticides, polychlorinated biphenyls (PCB) and methyl mercury are still performed using packed columns and the preparation and test of a suitable column will be described below.

60 Coating the support material for DDT compounds and PCB analyses

The stationary phase recommended for analyses of PCB and DDT compounds is a mixture of two silicone oils, SF96 (methyl silicone) and QF1 (fluoropropyl silicone). These are applied on the support, Chromosorb W (a diatomaceous earth material), which is delivered acid washed (AW) and deactivated with dimethyldichlorosilane (DMCS). For columns with internal diameter of 1-3 mm, the particle size of the support should be 100-120 mesh. To prepare 5 g filling material with 2.8% QF1 and 0.6% SF96, the following procedure is recommended:

1. Dissolve 140 mg QF1 in 20 ml acetone.
2. Dissolve 30 mg SF96 in 20 ml dichloromethane.
3. Mix the two solutions from 1 and 2.
4. Place 5.0 g Chromosorb W (AW, DMCS, 100-120 mesh) in a round bottom flask and add the combined stationary phase solutions from 3.
5. Connect the flask to a water jet air pump and evaporate the solvents while the flask is shaken carefully. A water bath or a heat lamp speeds up the evaporation. When the material is damp, it is placed in an oven (~100°C) to evaporate the solvent residues.

61 Preparing the column for DDT compounds and PCB analyses

The materials used for the column tubes are either stainless steel or glass. The less fragile steel is catalytically more active than glass and the latter material is therefore used in columns for pesticide analyses. The following procedure describes the cleaning and filling of glass columns.

1. Fill the column with concentrated HCl and leave it for one hour.

2. Wash with water, then with acetone and finally with toluene.

3. Fill the column with a toluene solution of hexamethyldisilazane (HMDS, 10%). Warning: use fume hood and do not touch the HMDS reagent!

4. Wash the column with toluene, then with methanol and finally with acetone.

5. Dry the column either with air, using a water jet air pump, or in an oven.

6. Put about 10 mm glass wool in the outlet end of the column and weigh the empty column.

7. Attach a funnel to the inlet of the column and fill at least half a coil with the filling material before the other end is connected to a vacuum pump (water jet air pump).

8. Fill the column by gentle tapping. If the filling material gets stuck before the column is filled, disconnect the water jet air pump and put the column in an oven (~100°C) for a few minutes and then continue with the warm column.

9. When the column is full, put about 10 mm glass wool in the inlet end.

10. Weigh the filled column and label it.

The column has to be conditioned before it is connected to the detector. Connect the inlet to the injector in a chromatograph (but not to the detector) and apply a carrier gas flow of about 20 ml/min and heat the column to allowed maximum temperature for the stationary phase (for the QF1-SF96 columns: 220°C). Preferably, the column should be conditioned overnight, but it can be used after one hour.

62 Conditioning columns for methyl mercury analyses

All-glass columns of 1.8 m length (inner diameter: 1.8 mm) are filled with 5% Carbowax monostearate on acid-washed silanized Chromosorb W. Apply a carrier gas flow of 30 ml/min and heat the column to the allowed maximum temperature of 150°C.

63 Column test

When the column has been connected to the detector, the carrier gas flow is set to 20 ml/min for a column with 2 mm internal diameter. The column performance is then measured by the 'number of theoretical plates' for a specific compound and can be done according to the following procedure.

1. Set injector and detector temperatures at 200 and 300°C respectively, and the column oven temperature at 180°C.

2. Inject p,p'-DDT or the methyl mercury standard, respectively, and measure their retention times (t_r). Adjust the column temperature to get about 20 minutes retention.

3. Measure the width of the DDT-peak or the methyl mercury peak respectively at its half height ($b_{\frac{1}{2}}$) in minutes and the retention time (t_r) also in minutes.

4. Calculate the number of theoretical plates with the formula

$$N = 5.54 \left(\frac{t_r}{b_{\frac{1}{2}}}\right)^2$$

5. A measure independent from column length is the height equivalent to a theoretical plate (HETP):

$$HETP = \frac{L}{N}$$

where L is the column length.

3.2 ELECTRON CAPTURE DETECTOR

64 A number of methods have been used to detect the compounds eluated from GC columns. Among the 'universal' methods, thermal conductivity (TCD) and flame ionization (FID) have shown to be useful.

65 For some applications, detectors with a higher compound-specific response are used. Polyhalogenated substances can be detected due to their ability to capture free electrons. The electron capture detector (ECD) thus gives good response for the chlorinated pesticides, PCB and methyl mercury. A few other types of compounds, including metal-organic and strongly conjugated substances can be detected with the ECD too. Other compounds in the sample give no or even negative response in this detector.

66 The procedures taking place in the ECD are not fully known yet. A simplified model is that β-particles from the radioactive source (^{3}H or ^{63}Ni) collide with nitrogen atoms in the carrier gas. Secondary, much slower electrons are then expelled and these give a current in an applied electric field. When a compound, capable to withdraw these electrons, is eluated from the column, the current decreases and this is registered on a strip chart recorder.

67 The signal thus measured is proportional to the concentration of electron-withdrawing compounds in the detector to a certain limit. When a considerable part of the electrons is captured, the probability for a molecule to find an electron is diminished and the proportionality is lost. This means that the response curve (response as function of the injected amount) will be linear for low amounts and then curve off (Fig. 5).

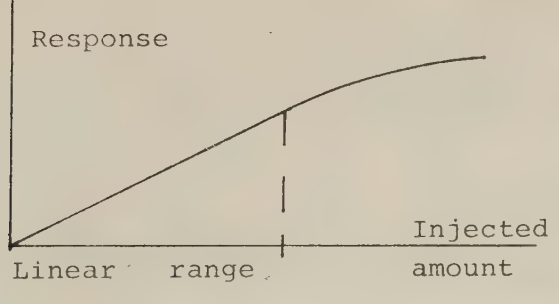


Figure 5. Response curve of ECD versus injected amount of eluated compound

68 The linear range for the ECD differs between instruments but covers normally from the detection limit to 100 - 10 000 times the detection limit. It is therefore essential to know the shape of the calibration curve and it can be constructed after injection of known amounts of the compound of interest. The samples are then diluted/concentrated to give responses within the linear range.

3.3 DETERMINATION OF CHLORINATED HYDROCARBONS

69 The method described has been used since 1967 without major changes for the analysis of DDT (I), DDD (II), DDE (III), HCB (IV), some HCH-isomers (V) and PCB (VI) (Fig. 6). The accuracy of the procedure has been verified in several international calibration exercises.

70 Chemicals

Acetone (purum), distilled
n-hexane (purum), distilled
2,2,4-trimethylpentane (iso-octane) (purum), distilled
Sodium chloride
Potassium hydroxide
Diethyl ether
Sulphuric acid
Orthophosphoric acid
Fuming sulphuric acid
Ethanol (95.5% spectroscopic grade)

All reagents, unless specified, are of analytical grade and tested in blank procedures.

71 Internal standards

In order to increase the accuracy of the analysis, an internal standard of 1,1-dichloro-2,2-diphenylethene (0.01 ng/µl) (VII) and 2,5,2',6'-tetrachloro-biphenyl (0.025 ng/µl (VIII) in iso-octane is used both as a solvent for the extracted fat and for the standards (Fig. 6). If further dilutions are necessary, these should be made with the internal standard solution.

72 Preparation of the internal standard

Stock solution: In a 100 ml volumetric flask, 100 mg of 1,1-dichloro-2,2-diphenylethene and 250 mg of 2,5,2',6'-tetrachlorobiphenyl is weighed on an analytical balance with an accuracy of 0.1 mg. The flask is placed on a balance with a reading of 1 mg and 69.2 g (100 ml) of iso-octane added.

Dilution 1 (10 and 25 µg/ml): In a 100 ml volumetric flask, 0.692 g of the stock solution is weighed, and the flask filled with iso-octane 69.20 g (100 ml).

Dilution 2 (0.01 and 0.025 µg/ml): This solution is made in the same way as above with 0.692 g (1 ml) of dilution 1 to one litre of iso-octane.

73 Extraction and fat isolation

1. Two separatory funnels (150 ml) are placed one above the other in a rack, the upper one having a G1 glass filter disk (Fig. 7).

2. Acetone (35 ml) and hexane (10 ml) are poured in the upper funnel, and in the lower funnel 50 ml of a solution of sodium chloride in orthophosphoric acid (11.7 g NaCl in 1 litre of 0.1 M orthophosphoric acid).

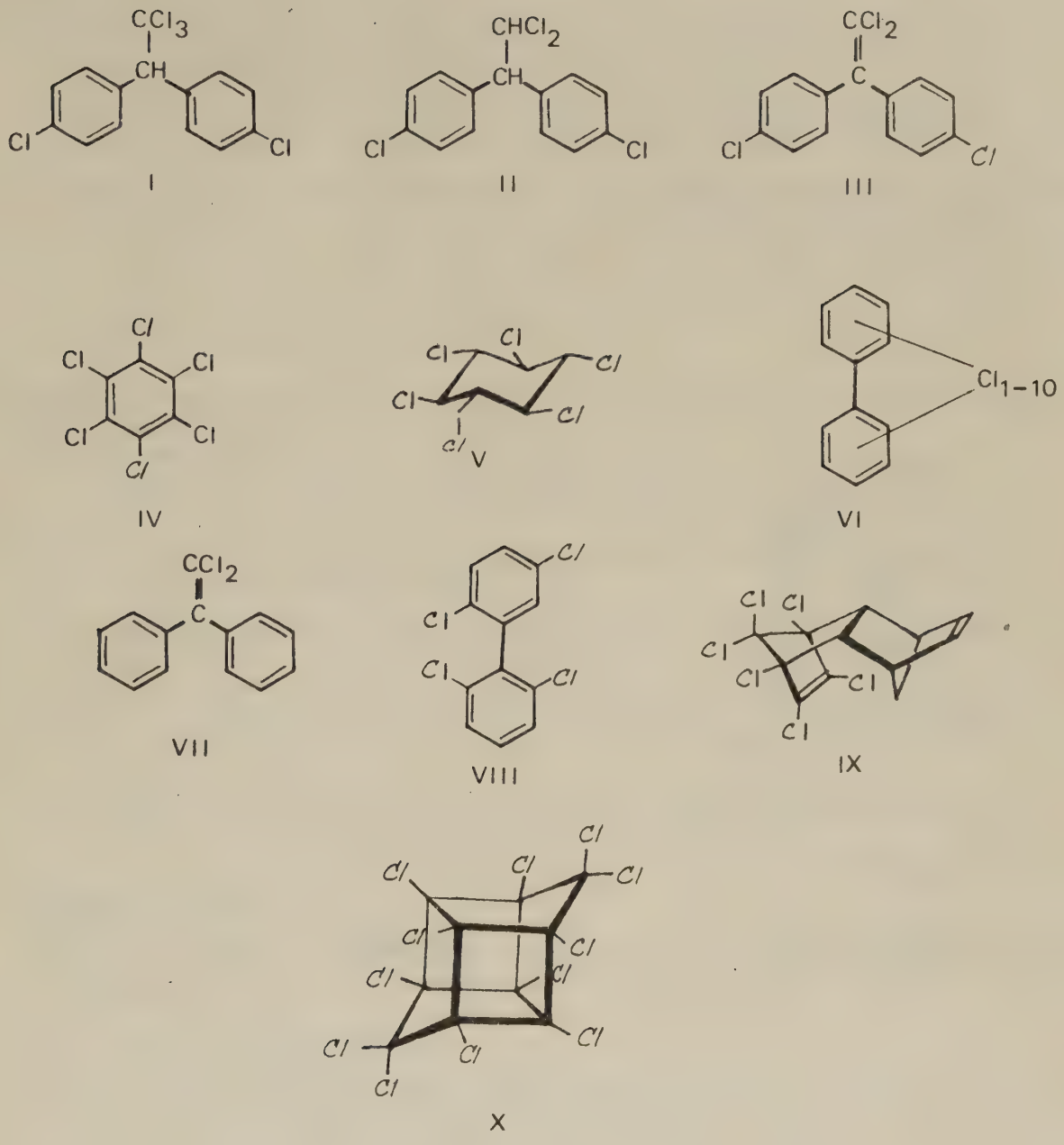
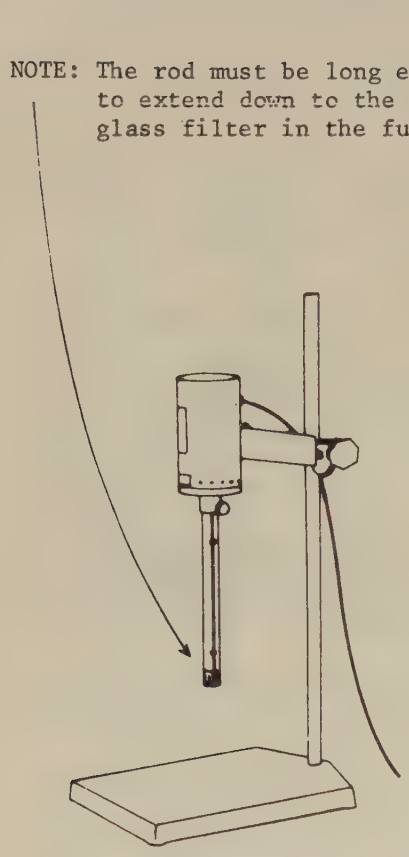


Figure 6. Formulas of several chlorinated hydrocarbons and internal standards

I	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (p,p'DDT)
II	1,1-dichloro-2,2-bis(p-chlorophenyl)ethane (p,p'DDD)
III	1,1-dichloro-2,2-bis(p-chlorophenyl)ethene (p,p'DDE)
IV	hexachlorobenzene (HCB)
V	γ-hexachlorocyclohexane (γ-HCH, lindane)
VI	polychlorinated biphenyl (PCB)
VII	1,1-dichloro-2,2-diphenylethene
VIII	2,5,2',6'-tetrachlorobiphenyl
IX	1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4:5,8-dimethano-naphthalene (aldrin)
X	dodecachlorooctahydro-1,3,4-metheno-2H-cyclobuta(c,d)pentalene (mirex)



'Ultra Turrax' homogenizer

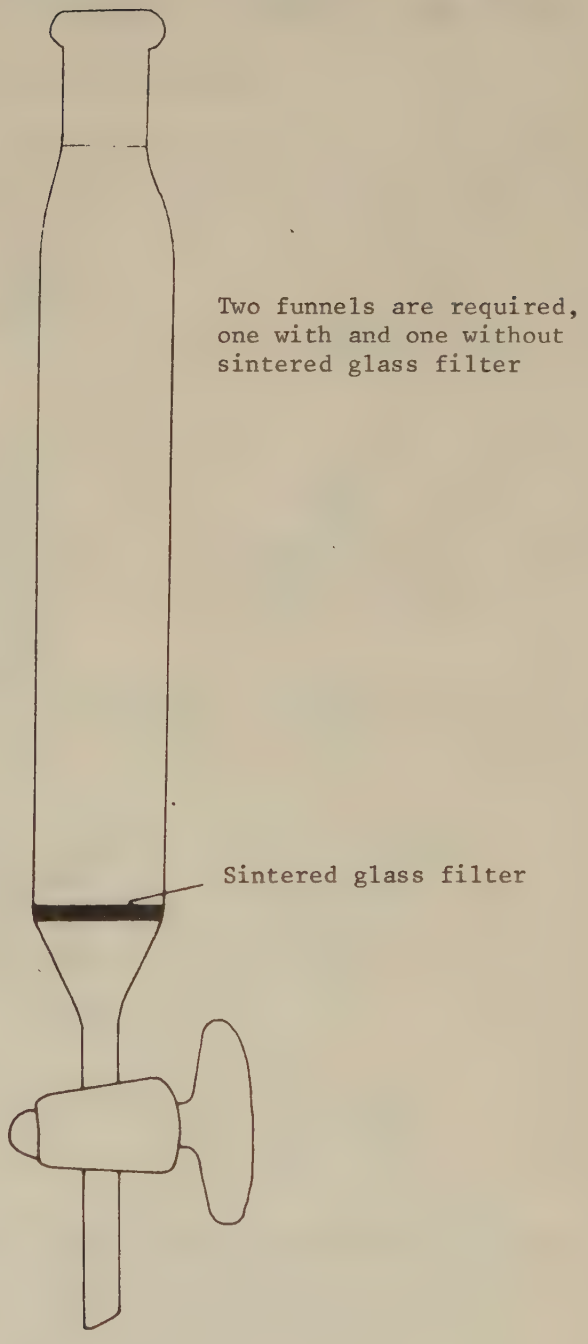


Figure 7. Equipment used for extraction of fish samples

3. The biological sample (approximately 10 g) is transferred into the upper funnel and homogenized with an insertable homogenizer (Fig. 7).

4. The solvents are passed into the lower funnel and a slight nitrogen gas pressure is applied to force the liquid through the glass filter.

5. The remaining tissue is stirred with a 9 : 1 mixture of hexane/diethyl ether (25 ml).

6. After five minutes, the liquid is passed into the lower funnel as above.

7. Finally, the sample is rinsed with a new portion of the hexane diethyl ether mixture (25 ml).

8. After shaking the funnel, the phases are allowed to separate and the lower aqueous layer is passed into a beaker (100 ml).

9. An Erlenmeyer flask (100 ml) is weighed (accuracy of 0.1 mg) and the upper organic fat extract is decanted into it.

10. The aqueous phase is poured back into the separatory funnel and re-extracted with hexane (10 ml).

11. After separation, the hexane is transferred to the E-flask. The flask is placed in a water bath and the temperature is raised to 70°C.

12. When the solvents have evaporated, acetone (10 ml) is added and evaporated. (Any water remaining in the fat will disappear azeotropically with the acetone).

13. Finally, when the E-flask has equilibrated at room temperature near the balance, the fat weight is determined (accuracy 0.1 mg).

74 Clean-up procedure

The fat is dissolved in the internal standard solution (about 100 mg/ml). If necessary, an ultrasonic bath is used to accomplish solution.

A. Sulphuric acid treatment

1. One ml of the fat solution is transferred into a test tube (8 ml) containing concentrated sulphuric acid (4 ml).

2. The test tube is closed with a teflon-lined screw cap and turned upside down carefully twenty times (do not shake).

3. The tubes are centrifugated, then the iso-octane is ready for gas chromatographic analysis.

B. Potassium hydroxide treatment

1. Two pellets of potassium hydroxide are dissolved in 95.5% ethanol (2 ml) together with water (0.1 ml).

2. Two ml of the fat solution are transferred to the tube which is sealed and placed in a water bath at 50°C for 30 minutes.

3. Add 4 ml of a solution of sodium chloride in orthophosphoric acid (11.7 g NaCl in 1 litre of 0.1 M orthophosphoric acid).

4. The tube is shaken and centrifugated; then the upper iso-octane layer is ready for analysis.

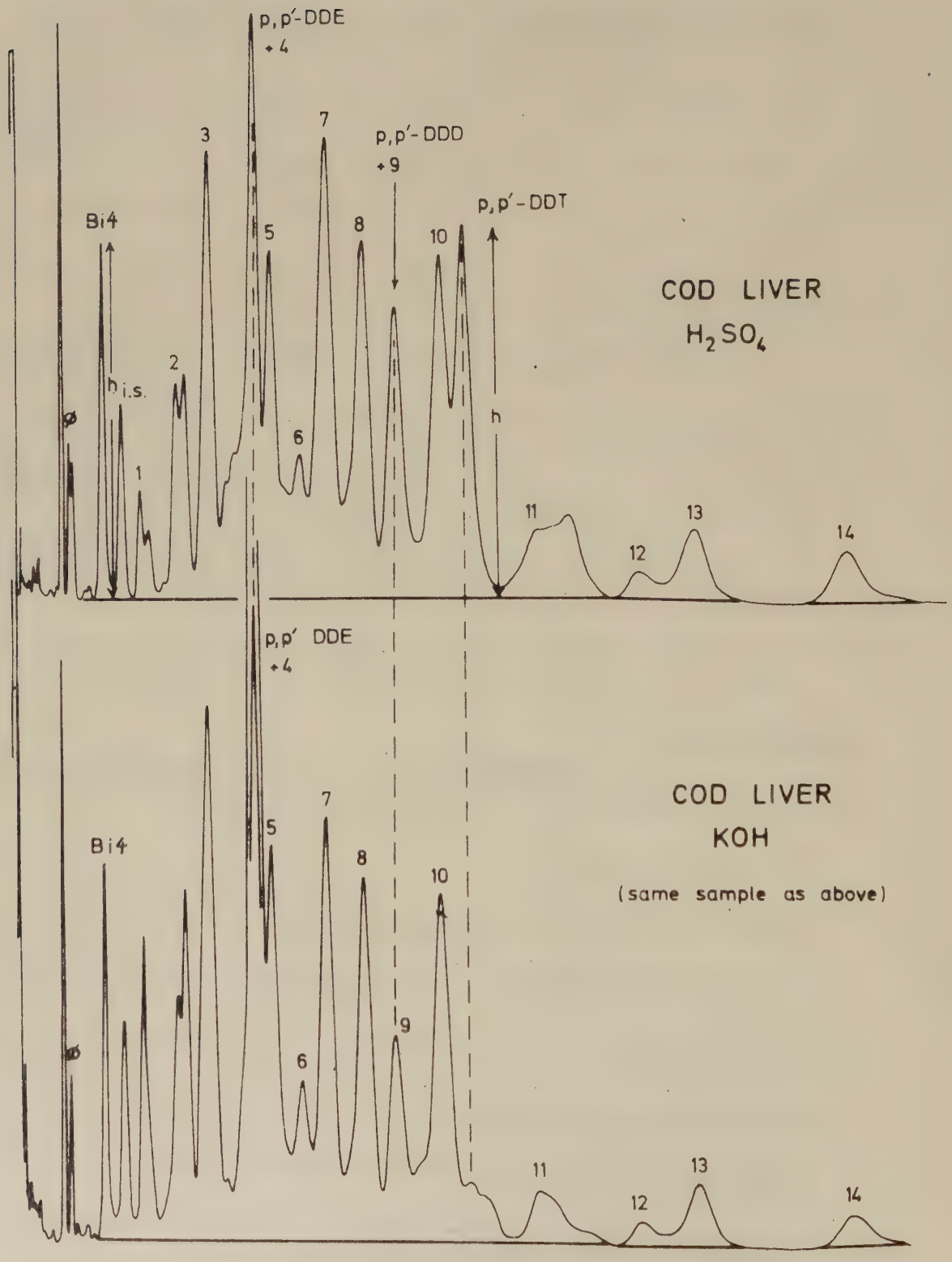


Figure 8. Chromatograms of a cod liver sample

75 Gas chromatographic determination

The samples should be analysed on a gas chromatograph equipped with an electron capture detector. The glass column should be filled with acid-washed and dusted Chromosorb W, 100-120 mesh, coated with 2.8% QF1 and 0.6% SF96 (paragraph 60). The column is normally programmed for 170-210°C, at a rate of 1°C/minute, to give p,p'-DDT a retention time of approximately 20 minutes. The injector is held at 200°C and the detector at 300°C. The carrier gas flow is set to 20 ml/minute.

76 Standard solutions

Two standard solutions containing pesticides and some metabolites of these are used. The concentrations and relative retention times compared to DDE are presented below (see Fig. 8).

		Figure 6	ng/µl	R_{DDE} (x100)
GLC standard A				
hexachlorobenzene	HCB	IV	0.05	23
1,1-dichloro-2,2-diphenylethene*	Ø	VII	0.01	27
γ-hexachlorocyclohexane (lindane)	γ-HCH	V	0.06	36
β-hexachlorocyclohexane	β-HCH		0.10	39
aldrin	Al	IX	0.05	53
o,p'-DDE			0.10	81
p,p'-DDE	DDE	III	0.20	100
dieldrin	Di		0.05	119
mirex		X	0.20	186
GCL standard B				
α-hexachlorocyclohexane	α-HCH		0.03	28
2,5,2',6'-tetrachlorobiphenyl*	Bi4	VIII	0.025	43
heptachlor			0.04	44
heptachlor epoxide			0.08	84
o,p'-DDT			0.2	115
p,p'-DDD		II	0.2	127
p,p'-DDT		I	0.2	142

*Internal standard

Other standard solutions contain different technical PCB mixtures with 42, 54 and 60% of chlorine (VI). When 1 ng Clophen A50 (54% chlorine) is chromatographed as described above, 14 major peaks will appear (see Figure 8), which correspond to the following amounts of PCB:

Peak	ng	Major components
1	0.074	2,3,2',5'-tetrachlorobiphenyl
2	0.110	2,5,3',4'-tetrachlorobiphenyl
3	0.169	2,5,2',4',5'-pentachlorobiphenyl
4	0.058	2,3,2',4',5'-pentachlorobiphenyl
5	0.115	2,3,2',3',4'-pentachlorobiphenyl
6	0.053	3,4,2',3',6'-pentachlorobiphenyl
7	0.100	3,4,2',4',5'-pentachlorobiphenyl
8	0.127	2,4,5,2',4',5'-hexachlorobiphenyl
9	0.036	3,4,2',3',4'-pentachlorobiphenyl
10	0.080	2,3,4,2',4',5'-hexachlorobiphenyl

Peak	ng	Major components
11	0.022	2,3,4,2',3',4'-hexachlorobiphenyl
12	0.025	3,4,2',3',4',5'-hexachlorobiphenyl
13	0.020	2,4,5,2',3',4',5'-heptachlorobiphenyl
14	0.010	2,3,4,2',3',4',5'-heptachlorobiphenyl
	0.999	

77 Standard stock solutions

Stock solution 1: 100 mg of each standard substance are weighed into a 100 ml volumetric flask and dissolved in 100 ml as above by means of the internal standard solution (dilution 2). The stock solution is stored in sealed glass ampoules.

Stock solution 2: Aliquots of the different stock solutions are weighed into a flask and diluted with internal standard solution (dilution 2) to 69.2 g (100 ml). Aliquots are calculated to result in concentrations 100 times higher than shown in paragraph 76 (standard solutions), e.g. 0.346 g (0.5 ml) of HCB stock solution, etc.

GLC standard solution: The solution is made from 0.692 g (1 ml) of stock solution 2 diluted with internal standard solution (dilution 2) to 69.2 g (100 ml).

78 Quantification

The extracts from clean-up A and B are chromatographed as well as standards and a solvent blank. The stability and the conversions of the different components according to the clean-up method are summarized in Table II. β- and γ-HCH, HCB, p,p'-DDE and p,p'-DDT are quantified on the basis of chromatogram A, PCB on the basis of chromatogram B, and dieldrin, o,p'-DDT and p,p'-DDD on the basis of the difference between chromatogram A and B (see Figure 8). Interference between p,p'-DDT and some PCB components sometimes occur in the chromatograms. This will be obvious by comparison of chromatograms A and B. Quantification of p,p'-DDT will then be based on the difference of the corresponding peaks in A and B. p,p'-DDE will overlap with peak 4 in the PCB pattern but this is a minor problem as this PCB component contributes very little to the total PCB component in biological material.

79 Calculation

After comparison of the retention times of peaks in the sample chromatogram to those in the corresponding standard, the peak heights are measured and related to the peak height in the standard, the residue is quantified according to the formula:

$$\frac{V}{M} \cdot \frac{h \cdot h'_{is}}{h_{is} \cdot h'} \cdot c = \text{result in } \mu g/g \text{ (ppm) on fresh weight basis}$$

V = total extract volume (ml)
M = fresh weight of the sample (g)
h = peak height of the compound in the sample (mm)
h' = peak height of the compound in the standard (mm)
h_{is} = height of the internal standard peak in the sample (mm)
h'_{is} = height of the internal standard peak in the standard (mm)
c = concentration of standard (µg/ml)

If no internal standard is used, peak heights of internal standards are replaced by injected volumes.

To calculate the result on fat weight basis: $\frac{a \cdot M}{m} = b$

a = result from fresh weight calculation
m = fat amount (g)

Table II

Stability of different compounds according to clean-up method

Compound	Clean-up		Transformation product
	A($H_2SO_4 \cdot SO_3$)	B(KOH/EtOH)	
HCB	+	+	
1,1-dichloro-2,2-diphenyl-ethene (internal standard)	+	+	
γ-HCH	+	-	
2,5,2',6'-tetrachlorobi-phenyl (internal standard)	+	+	
β-HCH	+	-	
aldrin	+	-	
p,p'-DDE	+	+	
dieldrin	-	+	
o,p'-DDT	+	-	o,p'-DDE
p,p'-DDD	+	-	p,p'-DDMU
p,p'-DDT	+	-	p,p'-DDE
PCBs	+	+	

+ Stable against treatment
- Not stable against treatment

3.4 SEPARATION OF CHLORINATED TERPENES AND CHLORDANE-RELATED COMPOUNDS FROM PCB

80 Two types of chlorinated hydrocarbons which can interfere with the analyses of PCB and DDT are chlorinated terpenes (camphechlor, toxaphene) and chlordane-related compounds. Both these types are very complex mixtures and it is impossible to improve the resolution of the GC column to separate all the individual isomers from PCB compounds.

81 There is no perfect method available to separate PCB from all components in Toxaphene. The method described below will, however, give about 90% of Toxaphene and chlordane in a PCB-free fraction.

82 The fat, extracted as described elsewhere in this manual, is dissolved in n-hexane (about 100 mg per ml) and shaken with sulphuric acid (98%) portions until the acid is colourless. The extract (in 0.5 ml) is applied to a silica gel column (1 g slurry-packed Kieselgel 60, 0.063 - 0.200 mm, Merck, activated at 250°C for 2 hours, deactivated with 3% water), which is eluated with 7 ml n-hexane (fraction 1) and then 10 ml 25% diethyl ether in n-hexane (fraction 2).

83 Fraction 1 contains hexachlorobenzene (HCB), DDE and PCB. These compounds can be determined by GC of this fraction after appropriate concentration (or dilution). Fraction 2 contains the HCH (hexachlorocyclohexane) isomers, DDD, DDT and most of the toxaphene and chlordane components. The interferences from DDD and DDT can be overcome by the following oxidation procedure.

84 Sulphuric acid (98%, 10 ml) is placed in a test tube with a teflon-line screwcap. Potassium dichromate (p.a., 1 g) is added and the tube shaken for 1 minute. Fraction 2, concentrated to 1 ml, is added and the tube shaken for 30 minutes (caution: the tube initially becomes hot). The hexane phase is transferred to another tube and shaken with sulphuric acid (98%) prior to GC analysis.

85 We do not know of any good methods to separate the chlorinated terpenes from the chlordane-related compounds. This, and the lack of reference compounds, makes the quantification of the chloroterpenes very difficult. Today, we can just estimate the amounts of these compounds by comparisons with known amounts of the technical products.

3.5 DETERMINATION OF METHYL MERCURY

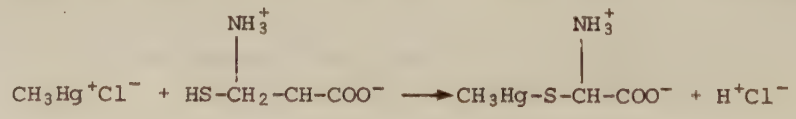
86 The biological effects of methyl mercury are well known. In contrast to the accumulating fat-soluble chlorinated pesticides, such as DDT, the methyl mercury accumulates because it is chemically bound to sulphur in the protein.

87 The first step in the analysis is consequently to break the bond between the methyl mercury and sulphur-containing amino-acids (such as cysteine) in the proteins. This is achieved by means of a strong mineral acid:

$$CH_3Hg\text{-}S\text{-protein} + H^+X^- \longrightarrow CH_3Hg^+X^- + HS\text{-protein}$$

X = halogen

88 Hydrochloric acid is normally used and the lipophilic methyl mercury chloride formed is then extracted into toluene. However, this solution is not sufficiently clean for gas chromatographic analysis. The methyl mercury chloride is therefore converted into an aqueous complex (here cysteine hydrochloride is used as the complexing agent) and the toluene phase is discarded.



89 This complex is broken with hydrochloric acid and the methyl mercury is transferred as the chloride into a new portion of toluene and the extract is injected into a gas chromatograph. The proper choice of stationary phase in the gas chromatographic column is essential and usual Carbowax 20M is chosen.

90 Chemicals

Toluene
Concentrated hydrochloric acid
Sodium sulphate
Cysteine hydrochloride
Methyl mercury dicyandiamide

91 Standard solution in toluene (1 µg Hg/ml)

Dissolve 15.1 mg methyl mercury dicyandiamide in 75 ml of water. Add 25 ml of concentrated hydrochloric acid. Shake the mixture with 100 ml toluene. Dilute 1 ml of the toluene phase in 99 ml toluene.

92 Extraction procedure

1. Homogenize 7 g of the sample with 2 ml water and transfer the suspension to a 8 ml test tube (screw cap and teflon disc).

2. Rinse the homogenizer and the homogenization tube with 1 ml water. Shake vigorously the combined portions with 1 ml concentrated hydrochloric acid and 3 ml toluene for 5 minutes.

3. Centrifugate, then remove with a Pasteur pipette the clear upper phase into a test tube.

4. Measure 2 ml of the toluene phase into another test tube, add 4 ml of a 1 percent cysteine chloride solution and shake vigorously for 2 minutes.

5. Centrifugate and transfer 3 ml of the aqueous phase to another test tube, add 1.0 ml concentrated hydrochloric acid and 2 ml toluene.

6. Shake for 2 minutes, centrifugate. The toluene phase is ready for injection into the gas chromatograph.

93 Gas chromatographic determination

The samples should be analysed on a gas chromatograph equipped with an electron capture detector. The glass column should be filled with 5 percent Carbowax 20M on acid-washed silanized and dusted Chromosorb W 100-120 mesh. A column temperature of 130°C gives a retention time of 3.5 minutes for methyl mercury chloride. The injector and the electron capture detector temperatures are maintained at about 160°C.

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